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Raw Milk as a Potential Source of Some Zoonotic Bacterial Diseases in Ismailia, Egypt

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Abstract: The bacterial contamination of milk from the affected dairy animals makes it unhealthy for human consumption. The objective of this study was to estimate the prevalence of *Streptococcus agalactia*, *S. uberis*, *Escherichia coli*, *Aeromonas spp*. and *Listeria monocytogenes* in milk collected from small dairy herds and supermarkets in Ismailia, Egypt. A total of 112 milk samples were collected from the entire milking of 80 of dairy household bovines and 32 milk samples collected from retail supermarkets. In addition, stool swabs from consenting animal owners and milkers were collected. Milk samples were submitted for bacteriological and molecular examination. Results revealed that the overall prevalence of contaminates as *S. agalactia*, *S. uberis*, *E. coli*, *Aeromonas spp*. and *L. monocytogenes* was 22.32%, 6.25%, 47.32%, 15.17% and0.89%, respectively. The isolation rate of the studied zoonotic bacteria from household milk samples was much higher than that detected in the supermarket milk samples. Results revealed that virulent *E. coli* O157: H7 strain was isolated and confirmed by PCR from 2 (1.79%) milk samples collected from household dairy bovines. Among 35 stool samples collected from animal owners and milkers, 8 (28.57%) was positive for *E. coli* spp., whereas 4 (11.42%) was positive for *Aeromonas spp*. However, *L. monocytogenes* and *E. coli* O157:H7 were not detected in stool samples. In conclusion, this study indicated a potential risk for human exposure through consumption of raw milk and dairy products processed from this milk. Milk from household dairy animals was more likely to provoke health hazards than milk sold in the supermarkets.

Key words: Milk · *Streptococcus* · *Listeria* · *Aeromonas* · Zoonoses

INTRODUCTION

The zoonotic bacteria; *Strepotococci*, *E. coli, L. monocytogenes* are known to infect dairy cows with clinical and subclinical infections [1, 2]. The presence of these opportunistic, pathogenic bacteria in milk has emerged as a public-health concern especially among individuals who consume raw milk and related dairy products [3, 4].

Streptococci are major human and animal pathogens divided into more than 40 subspecies and multiple groups [5]. Streptococci continue to be a major cause of subclinical mastitis in dairy cattle and a source of economic loss for the dairy industry [6]. *S. agalactiae* and *S. uberis* are major etiologic agents of clinical and subclinical mastitis and a potential contaminant of the milk. Streptococcal infections in humans are associated with scarlet fever, sore-throat, tonsillitis, bacterial endocarditis, rheumatic fever and pneumonia [7].

Enterotoxigenic *E. coli* (ETEC) strains cause watery diarrhea in animals and birds worldwide. *E. coli* contamination of food staffs is a matter of concern to public health, being a wide spread human foodborne pathogen [8]. Enterohemorrhagic *E. coli* (EHEC) is a subset of pathogenic *E. coli* that can cause diarrhea or hemorrhagic colitis in humans. Hemorrhagic colitis occasionally progresses to hemolytic uremic syndrome (HUS), an important cause of acute renal failure in children, morbidity and mortality in adults. On subspecies level, *E. coli* O157:H7 (*E. coli* O157) is an important cause

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of human diarrheal disease. Severe manifestations include (HUS) [9]. The infectious dose is very low, which increases the risk of disease. The reservoirs for EHEC are ruminants, particularly cattle and sheep, which are infected asymptomatically and shed the organism in feces. Humans acquire EHEC O157:H7 by direct contact with animal carriers, their feces and ingestion of raw food including milk [10].

Aeromonas is widely distributed globally in aquatic environments and associated with a variety of human infections, including gastroenteritis, soft tissue infection, septicemia, hepatobiliary tract infections and occasionally pleuropulmonary infections, meningitis, peritonitis and hemolytic uremic syndrome [11]. Although this pathogen could infect healthy persons, most infections were found in immune-compromised hosts, especially those with liver cirrhosis and malignancies [11, 12]. *Aeromonas* is not causing mastitis but usually contaminate milk during unhygienic milking, bad handling and improper storage conditions.

L. monocytogenes is a food-borne pathogen that may cause a severe, invasive illness with a corresponding mortality rate up to 30% in susceptible populations [13, 14]. The excretion of *Listeria* in milk may persist throughout lactation and contribute to an increased risk of milk product contamination [13].

Identification of bacterial pathogens in raw milk is regarded as the definitive identification of food poisoning sources. It also provides information important for prevention and control of these poisonings. Therefore, the objective of this study was to investigate the prevalence of some zoonotic bacteria in raw milk collected from small household dairy collection and supermarkets in Ismailia, Egypt by using conventional culture and PCR tests. In addition, this study aimed to assess the public health impacts of contamination of raw milk with these bacterial agents.

MATERIALS AND METHODS

Study Area and Sample Collection: A total of 112 milk samples were included in this study. Milk samples from smallholder farms (N=80), having between one to five lactating animals and were randomly selected for this study in the Ismailia city, Egypt, between March 2012 and June 2013. The dairy animals were maintained under suboptimal conditions, with milking being done by hand. Milk samples (250ml) collected from all animals (bulk milk) from the same house considered as one sample.

In addition, 32 milk samples were collected from supermarkets in Ismailia city. The supermarket milk was sold in chilling tanks with continuous automatic stirring. Samples of the supermarket milk were provided from large dairy farms (more than 50 diary animals) in refrigerated tanks during transportation. These farms were automatically milked following proper sanitation protocols. Transportation of milk was done using chilling tanks. The milk in the bulk tank was agitated for 5 to 10 minutes and approximately 250 ml of milk was taken from the top of the bulk tank. Samples were immediately placed on ice and transported to the laboratory.

Human Samples and Questionnaires: In order to identify potential risk of transmission of some food poisoning bacteria by milk, 80 household animal owners were agreed for oral consent to take part in this study. Attitudes and practices related to milk-transmitted diseases were assessed by investigating the management of livestock and processing and consumption of dairy products.

Stool samples were collected from 35 animal owners (median age, 28 years), who were in contact with the animals and agreed to take part in this survey. These samples were transferred to the laboratory on ice with minimal delay.

Bacteriological Examination: Milk samples and stool samples received from the owners were subjected to bacteriological examination as well as PCR. Samples were cultured and identified according to [15]. Isolates were confirmed by biochemical tests and PCR. Ten ml of the milk samples were centrifuged in a centrifuge tube at 5000 rpm for five min, a loopful of the supernatant and sediment was inoculated onto 10 ml of buffered peptone water and incubated for 24h at 37°C.

For bacteriological culture of streptococcus species, 0.1ml from each pre-enriched sample was streaked onto Steptococcus selective agar base (HIMEDIA). The plates were incubated for 24 h at 37°C. Suspected colonies were characterized by β-hemolysis. *S. agalactiae* was identified by Gram stain, negative catalase test, negative esculin hydrolysis, positive CAMP test and positive reaction to Lancefield group B antiserum. *S. uberis* growth of red colonies on SlaBa plates (Slanetz & Bartley Medium, Oxoid Ltd., Basingstoke, England) was evaluated.

For E. coli Isolation, a loopful of the pre-enriched sample was added to broth cultures were plated onto MacConkey agar (Oxoid) and incubated for 24-48 h at 37°C. The colony indicated for *E. coli* by MacConkey

Table 1: Sequences, amplicon size, targeted genes and annealing temperatures of the PCR primers

Microorganisms	Sequence (5'-3')	Amplicon size (bp)	Target genes	Annealing tem	References
S. agalactiae	F: 5'-TTTGGTGTTTACACTAGACTG-3'				
	R: 5'-TGTGTTAATTACTCTTATGCG-3'	120	16S rRNA	57	[17]
S. uberis	F: 5'-TCGCGGTATTGAAAAAGCAACAT-3'				
	R: 5'-TGCAATAATGAGAAGGGGACGAC-3'	400	cpn60	60	[18]
E. coli spp.	F:5-ACCTGCGTTGCGTAAATA-3.				
	R:5-GGGCGGGAGAAGTTGATG-3.	670	gadA/B	60	[19]
E. coli O157: H7 eaeAO157:F	F:5-AAG CGA CTG AGG TCA CT-3				
	R: 5-ACG CTG CTC ACT AGA TGT-3	450	eaeAO15:7	50	[20]
Aeromonas spp.	F:5-TCATGGCTCAGATTGAACGCT-3				
	R: 5-CGGGGCTTTCACATCTAACTTATC-3	599 bp	16S rRNA	56	[21]
L. monocytogenes	F: 5'-AACCTATCCAGGTGCTC-3'				
	R: 5'-CTGTAAGCCATTTCGTC-3'	267	*hlyA* gene	55	[22]

agar were further subcultures onto Eosin Methylene Blue Agar (EMB) agar, followed by incubation at 37°C for 24 h. *E coli* colonies are colored blue/green. E coli isolates were confirmed by PCR and surveyed for O157: H7 serotype by PCR reaction. Suspected *E. coli* spp. based on colony morphology on the selective media, were identified by biochemical tests.

Isolation and identification of *Aeromonas* spp. from the collected samples were adopted. A loopful of the pre-enriched sample was added to tubes containing 10 ml tryptone soy broth (Difco) supplemented with 10% ampicillin followed by incubation at 37°C for 24-48 h. A loopful from each tube was streaked over the following media trypticase soya agars (Difco), RS agar (Difco), MacConkey agar (Oxoid) and Aeromonas base medium (Difco) and incubated at 37 °C for 24-48 h. The suspected purified colonies were screened using biochemical tests according to Abbott *et al.* [16].

For *L. monocytogenes* detection in milk samples, microbiological standard methods were used. Enrichment was done by adding 1 ml of the milk sample into 9 ml of *Listeria* Enrichment Broth (LEB) (Himedia) and incubated at 30°C for 2-7 days. A loopful from the LEB culture was streaked onto OXFORD media (Himedia) supplemented with Listeria Oxford supplement (Himedia) and incubated for 24-48 h at 35°C. Grey colonies surrounded by black zones were presumed to be *Listeria*. The suspect purified colonies were screened using biochemical tests.

Reference strains of the examined bacteria were included as positive controls.

DNA Extraction and PCR Amplification: DNA extraction was performed according to the manufacturer guidelines using Bacterial DNA Extraction Kit (Spin-column) (BioTeke Corporation). Amplification reactions were carried out with 5 µl of boiled bacterial suspensions, 5 µl of 5X Taq Master/ high yield (Jena Bioscience, GMBH, Germany) containing Thermostable DNA Taq polymerase buffer, dNTPs, (NH4) 2SO4, MgCl2 and two pairs of primers 50 pmol. Double-distilled water was added to bring the final volume to 25 µl. The PCR assays were performed using a Thermal Cycler (Eppendorf). The primers were ordered from Operon Company, (Operon, Japan) as nucleotide sequence.

The amplification procedure consisted of an initial denaturation step at 94 °C for 2 min, followed by 30 cycles with denaturation at 94 °C for 1min, annealing for 1 min at different temperatures according to the targeted gene as shown in Table 1 and extension at 72 °C for 1 min. A final extension step was carried out at 72 °C for 5 min. Aliquots from amplification reactions were analyzed by 1.2% agarose gel electrophoresis and viewed and photographed under UV light using gel documentation system (Biospectrum UVP, UK). Products of the appropriate sizes were identified by comparisons with a 100-bp DNA ladder (Gibco). Different sets of the primers, targeted genes and annealing temperature were shown in Table 1. In each PCR run, a non-template control was included to detect possible external DNA contamination and control positive were used for confirmation.

RESULTS

Clinical Examination of Household Dairy Animals: All dairy cattle and buffalo included in this study were apparently healthy. The milk from these animals was normal shape and consistency without any signs of mastitis. Dairy animals were manually milked twice a day.

Isolation Rates of Some Bacterial Species among Milk Samples: As shown in Table 2, results revealed that the overall prevalence of milk contaminates by *S. agalactia, S. uberis, E. coli, Aeromonas spp*. L. monocytogenes was 22.32%, 6.25%, 47.32%, 15.17% and 0.89%, respectively.

Table 2: Detection rates of bacterial contamination of some zoonotic bacteria in milk samples

	Supermarket milk No= 32		Household milk No=80		Total No = 112	
Bacteria spp.	+ve	%	+ve	%	+ve	%
S. agalactiae	7	21.88	18	22.5	25	22.32
S. uberis	1	3.13	6	7.5	7	6.25
E. coli spp.	11	34.38	42	52.5	53	47.32
E. coli O157:H7	0	0	2	2.5	2	1.79
Aeromonas spp.	3	9.38	14	17.5	17	15.17
L. monocytogenes	0	0	1	1.25	1	0.89

Table 3: The rate of isolation of *E. coli, Aeromonas and L. monocytogenes* from human stool samples

	Stool samples (No=35)	
Isolated bacteria	No.	%
E. coli spp.	8	28.57
E. coli O 157:H7	0	0
Aeromonas spp.	4	11.42
L. monocytogenes	0	0

Regarding the milk samples collected from the supermarkets, out of 32 samples, (22.5%) *S. agalactiae*, (7.5%) *S. uberis*, (52.5%) *E coli*, (17.5%) *Aeromonas* spp. and (1.25%) *L. monocytogenes* was isolated and confirmed by PCR. Out of 80 milk samples of household dairy animals, 7 (21.88%) *S. agalactia*, 1 (3.13%) *S. uberis*, 11(34.38%) *E. coli* spp., 3 (9.38%) *Aeromonas s*pp. and 0% *L. monocytogenes* was isolated by conventional bacteriological examination and confirmed by PCR assays.

Prevalence of *E. coli* O157:H7 among the Examined Milk Samples: Results revealed that virulent *E. coli* O157: H7 was isolated and confirmed from 2 (1.79%) milk samples collected from household dairy cattle and buffalo. However, it was negative in supermarket milk samples examined by the same technique.

Human Samples: Among 35 stool samples collected from animal owners, 8 (28.57%) was positive for *E. coli* spp. whereas, 4 (11.42%) was positive for *Aeromonas spp*., However, none were positive for *L. monocytogenes* and *E. coli* O157:H7 strain by either assay.

Questioner Results: A total of 80 households individual animal owners were participated in a questionnaire. High percentage (68.75%) of households regularly sold their raw milk; whereas the other participants processed their milk into cheese, cream and/or butter. All participants boiled raw milk before consumption but no participants' boiled milk before processing it into other dairy products.

DISCUSSION

Milk is an important source of proteins, sugars, lipids and other nutrients for humans. However, these nutrients can also serve as substrates for pathogenic microorganisms. The consumption of homemade dairy products, especially cheeses, which made from raw milk, poses a serious risk to public health [3, 4, 23]. Our results revealed occurrence of some zoonotic bacteria in the raw milk. With regard to the risk of human exposure to infections via drinking milk, the results suggested that there was a high risk from milk consumption. Since all participants boiled their raw milk before consumption, the risk was minimal from liquid milk consumption. However, high risk mainly comes from consumption of the processed dairy products which consumed regularly in more than 80% of households, such as homemade cheese from raw milk.

Our results revealed higher bacterial contamination of milk collected from the household dairy bovine animals than milk collected from the supermarkets. This indicated higher potential risk of zoonotic bacterial infection through the consumption of raw milk and related homemade dairy products. This finding could be explained by the fact that hygienic standards of husbandry and milking process are suboptimal in the household animal collections. While milk sold in the supermarkets usually come from large dairy farms automatically milked with better hygienic measures. Farmers in Egypt are smallholders and the fresh milk is an important source of cash income. Milk is mainly sold locally in different sale channels directly to consumers, or through intermediaries [24]. According to [25], small herds are a majority in the developing countries. In these herds, animal health care is scarce because producers carry out neither preventive medicine nor a hygienic handling of milk during milking and milk contamination by many zoonotic pathogens is likely to occur. Similarly, in Mexico, family dairy herds or small-scale dairy enterprises contribute to the national milk production with values ranging from 35 to 40% [26].

Results of this study revealed a high detection rate of *E. coli, S. uberis* and *S. agalactiae.* These bacterial agents are the most leading causes of clinical and subclinical mastitis [1]. Varying prevalence was recorded in many studies [27, 28]. The high concentration of bacterial contaminants is an indication that the hygiene and safety of milk is compromised. The detection of same bacterial agents in human stool could not be related to milk consumption. Therefore, further investigation of these isolates by molecular analysis is needed to determine the human-livestock linkage.

Of contagious pathogens of the udder, *S. agalactiae* predominate in all regions of the world [7]. The high prevalence of *S. agalactiae* detected in this study indicated that subclinical mastitis still a major herd problem. Little is known about the likelihood of Group B Streptococcus (GBS) transmission between animals and humans. It has subsequently been suggested by several investigators that interspecies GBS transmission is not likely [29]. However, it was proposed that intra-species transmission GBS was proposed as a possible zoonotic infection, which has significant public health [7].

S. uberis is an environmental bacterium responsible for bovine mastitis. Our results were much lower than that detected in other studies accounting 11.1% of the cases in Sweden [30]. Regarding human infections, *S. uberis* is occasionally described as a human pathogen [23].

E. coli is an environmental bacterium responsible for bovine mastitis. *E. coli* has the ability to survive and grow up to 16 days in raw and pasteurized milk kept at 4C° [31]. *E. coli* were found in 61% of the herds [28]. In particular, E. *coli* O157:H7 is an important cause of human diarrheal disease. The infection risk is high because the infective dose of *E. coli* O157:H7 is low [32]. Detection of E. *coli* O157:H7 in raw milk in this study indicated a potential health hazards if this milk consumed raw or used directly for making soft cheese [33].

The excretion of *Listeria* in milk may persist throughout lactation and contribute to an increased risk of milk product contamination [13]. Although the detection rate of *L. monocytogenes* in row milk was very low in this study it is very serious to the consumers. Studies on presence of *L. monocytogenes* in raw milk, carried out in Europe, have shown that 2.5-6% of samples can be contaminated with *L. monocytogenes* [34].

In conclusion, this study indicated that there was a potential risk for human through consumption of raw milk and dairy products made from this milk. Milk from household dairy animals was more likely to provoke health hazards than milk sold in the supermarkets. Therefore, suitable processing parameters and post processing handling precautions should be done as control measures to minimize or eliminate the hazard associated with this risk.

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